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Activation of extracellular-regulated kinases by normal and mutant EGF receptors

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Abstract

Glioblastoma cells express a mutant EGF receptor (EGFRvIII) that has constitutive tyrosine kinase activity and enhances their tumorigenicity. Here we show that EGFRvIII promotes constitutive phosphorylation of extracellular regulated kinases (ERKs) in glioblastoma cells in the absence of EGF. EGFRvIII also promoted constitutive activation of phosphoinositide 3-kinase in these cells, as assessed by phosphorylation of protein kinase B/akt. As expected, phosphorylation of protein kinase B/akt was blocked by the phosphoinositide 3-kinase inhibitors wortmannin and LY294002. Less expectedly, we found that this treatment also blocked EGFRvIII-induced phosphorylation of ERKs. In contrast, ERK phosphorylation induced by EGF-activated normal EGF receptor in the same cells was largely unaffected by treatment with phosphoinositide 3-kinase inhibitors. This difference in behavior between the normal receptor and EGFRvIII was not due to differences in the levels of activated EGFRvIII and wild-type EGF receptor, as the two types of receptor were tyrosine phosphorylated to a similar extent under the experimental conditions used. EGFRvIII activation of ERKs was also sensitive to the phospholipase C inhibitor U73122, whereas ERK activation by normal EGF receptor was not. These results show that EGFRvIII and wild-type EGF receptor preferentially use different signaling pathways to induce ERK phosphorylation. The different mechanisms of ERK activation used by normal and mutant EGF receptors may be important in understanding the potent tumorigenic activity of EGFRvIII.

Keywords: EGF receptor; EGFRvIII; Extracellular-regulated kinase; Phosphoinositide 3-kinase; Phospholipase C; Glioblastoma

1. Introduction

A mutant EGF receptor, EGFRvIII, which lacks the regions coded for by exons 2–7 of the EGF receptor gene, has been detected in carcinoma of the brain, breast [1,2], lung [3], prostate [4] and ovary [2]. EGFRvIII has been studied in most detail in glioblastoma, where it is reported to be present in 24–52% of cases [1,5]. Expression of EGFRvIII is maintained when human glioblastoma cells are grown as xenografts, but is lost when the cells are grown in tissue culture [6]. When EGFRvIII is reintroduced into glioblastoma cell lines, it greatly enhances their tumorigenicity in vivo [7]. This correlates with both

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increased proliferation and decreased apoptosis within the tumors [8].

The molecular mechanisms by which EGFRvIII promotes aggressive in vivo growth of glioblastomas have not been fully elucidated. In glioblastoma cells, EGFRvIII has been shown to be constitutively phosphorylated on tyrosine residues [9]. Also, EGFRvIII has been shown to constitutively associate with the adapter proteins Shc and Grb2 and to increase levels of ras in the GTP-bound form [10]. In addition, there is evidence that EGFRvIII increases the activity of extracellular regulated kinases (ERKs) [11], which are widely recognized as having an important role in the regulation of proliferation and apoptosis [12]. However, the mechanism by which EGFRvIII activates ERKs has not been established. While a pathway in which ras activates a protein kinase cascade consisting of raf-1, MEK and ERK is well established [13], numerous questions remain about the regulation of this pathway, and the nature and role of alternate pathways leading to ERK activation. In particular, one question that has not been resolved is the role of phosphoinositide (PI) 3-kinase in regulating this pathway. There have been a number of reports that inhibitors of PI-3 kinase block ERK activation by tyrosine kinase receptors in some cell types, but not in others. With respect to the EGF receptor, it has been reported that in parietal cells the PI 3-kinase inhibitor wortmannin had no effect on EGF-induced activation of ERKs [14]. In COS cells, it has been reported that EGF-induced activation of ERKs is wortmannin-sensitive, but only when very low levels of EGF receptors are activated [15]. Again the exact mechanism of this inhibition is unknown, although there is evidence that it occurs both above and below the level of ras [15].

In this report we show that EGFRvIII induces the constitutive phosphorylation of ERKs in glioblastoma cells. This phosphorylation was blocked by two different PI 3-kinase inhibitors, and also by a phospholipase C inhibitor. However, ERK phosphorylation induced by normal EGF receptors in the same cells was not affected by these inhibitors. This difference was detected under conditions where levels of activated, tyrosine-phosphorylated normal EGF receptor and EGFRvIII were similar. Thus the two types of receptors use different mechanisms to activate ERKs.

2. Materials and methods

2.1. Antibodies and inhibitors

Phospho-p44/42 MAP kinase (Thr202/Tyr204) E10 monoclonal antibody, phospho-Akt (Ser 473) monoclonal antibody and PD98059 were from New-England Biolabs (Beverly, MA). Pan-ERK antibody and anti-phosphotyrosine antibody PY20 were from Transduction Laboratories (Lexington, KY). Akt1(C20) goat polyclonal antibody and EGFR(1005) rabbit polyclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Wortmannin, U73122 and U73343 were from Sigma-Aldrich Canada (Oakville, ON). LY 294002 was from Calbiochem-Novabiochem (La Jolla, CA). EGF was from Gibco-BRL (Rockville, MD).

2.2. Cell culture

U87MG glioblastoma cells and U87MGΔEGFR cells were obtained from Dr W. Cavenee (Ludwig Institute for Cancer Research, La Jolla, CA). U87MGΔEGFR were originally made by transduction of U87MG cells with a retroviral vector containing the coding sequence of EGFRvIII, followed by fluorescence-activated cell sorting for high expressors [7]. U87MGΔEGFR cells have normal p53, but are mutated in the PTEN tumor suppressor and at the INK4a locus, expressing neither $p16^{INK4a}$ nor $p19^{ARF}$ [16]. This set of mutations resembles those seen in primary tumor samples taken from de novo glioblastoma multiforme patients [17]. Cells were routinely cultured in DMEM supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum. For Western blot samples, cells were plated in medium containing 10% fetal calf serum at 2×10^6 per 100-mm plate or 3×10^5 per 35-mm plate, and then switched to medium containing 0.5% fetal calf serum the following day. Inhibitor treatments and collection of samples for Western blotting were carried out two days after plating.

2.3. Western blotting

Cells in monolayer culture were washed twice in ice-cold PBS and then lysed by the addition of hot

lysis buffer (5 mM Tris (pH 6.8), 4% sodium dodecyl sulfate, 2% glycerol, 0.2 M dithiothreitol). Samples were then scraped into microfuge tubes, boiled for 10 min, sonicated, and boiled again for 5 min. Protein concentrations were measured using the Bio-Rad Protein assay (Bio-Rad Laboratories, Hercules, CA). Samples were run on 12% sodium dodecyl sulfate–polyacrylamide gels and transferred to nitrocellulose. After transfer, blots were stained for total protein with a solution of 1% amido black in 50% methanol, 10% acetic acid. This served as a control that equal amounts of total protein were loaded and transferred per lane. Blots were then washed with H_2O, blocked with 5% skimmed milk powder in TBST (10 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20) for 1–2 h and incubated with the appropriate primary antibody at dilutions recommended by the supplier. Blots were then washed, incubated with second antibody (goat anti-mouse from Bio-Rad Laboratories) and washed again. Blots were developed using the LumiGLO chemiluminescence substrate kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

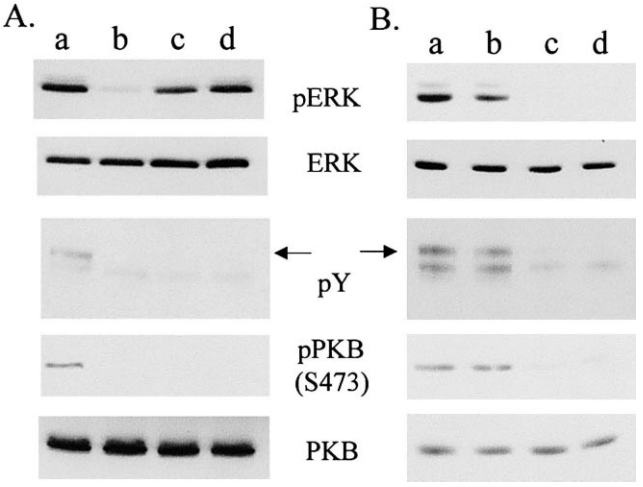

Fig. 1. (A) AG1478 treatment of U87MGΔEGFR and U87MG cells. U87MGΔEGFR (lanes a,b) and U87MG cells (lanes c,d) were treated for 2 h with either DMSO (lanes a,c) or 100 μM AG 1478 (lanes b,d). Total cell extracts were harvested and analyzed by Western blotting for either phosphorylated ERK (pERK), total ERK protein, phosphotyrosine, phosphorylated PKB or total PKB protein. DMSO at the concentrations used in this or the following experiments had no effect on pERK or ERK levels. (B) U87MGΔEGFR cells were treated with 0 μM (lane a), 0.1 μM (lane b), 1 μM (lane c) or 10 μM (lane d) AG1478 for 1 h and analyzed as above.

3. Results

3.1. EGFRvIII induces constitutive ERK phosphorylation

We first established that EGFRvIII induced constitutive phosphorylation of ERKs in glioblastoma cells. U87MGΔEGFR and U87MG cells were incubated overnight in 0.5% fetal calf serum and then treated for 1–2 h with the EGF receptor tyrosine kinase inhibitor AG1478 or DMSO as a control. AG1478 has been shown to be highly specific for the EGF receptor tyrosine kinase [18], and has also been shown to inhibit EGFRvIII tyrosine kinase activity [19]. Total cell lysates from treated cells were prepared as described in Section 2, and analyzed by Western blotting (Fig. 1). Blots were probed with an antiserum recognizing both ERK1 and ERK2 (pan ERK), and antiserum that recognizes the same proteins only when they are phosphorylated at threonine-202 and tyrosine-204 (numbering based on human ERK1 sequence). With pan ERK antiserum, a band was detected of the MW expected for ERK2, and with longer exposures a second slightly higher MW ERK1 band was detected. Levels of ERKs were unaffected by treatment with AG1478 and were the same in both U87MGΔEGFR and U87MG cells. When blots were probed with a monoclonal antibody specific for phosphorylated ERKs, a strong doublet was detected in U87MGΔEGFR cells. This intensity of this doublet was reduced to almost undetectable levels by treatment with AG1478. AG1478 was effective in this regard at concentrations as low as 1 μM (Fig. 1B) and inhibition was apparent after as little as 15 min of treatment (not shown).

Phosphorylated ERKs were also detected in U87MG cells. However, levels of phosphorylated ERKs in these cells were completely unaffected by treatment with AG1478. The ERK phosphorylation is therefore not due to autocrine activation of normal EGF receptor in these cells. Consistent with this, phosphorylated ERK levels were also unaffected by incubation of cells with an antibody that blocks binding of EGF and TGFα to wild-type EGF receptor (data not shown). Levels of phosphorylated ERKs in U87MG were on average slightly lower

than in U87MGΔEGFR cells, consistent with the work of Wu et al. which showed increased kinase activity of ERK1/2 in U87MGΔEGFR compared to U87MG cells [11]. However, this difference was quite small. Levels of phosphorylated ERKs in U87MG cells were invariably higher than in AG1478-treated U87MGΔEGFR cells. This may be due to antagonism of other ERK activation pathways by EGFRvIII. Montgomery et al. have reported that EGFRvIII expressed in mouse fibroblasts inhibits ERK activation by phorbol ester and serum [20], and provide evidence that the inhibition is due to increased phosphatase activity.

We also compared the effects of AG1478 on EGFRvIII tyrosine phosphorylation with the effects on ERK phosphorylation. Analysis of total cell extracts by Western blotting with antibody to phosphotyrosine showed a identified a band of 140 000 kDa. This band was absent in U87MGΔEGFR cells treated with AG1478 and in U87MG cells showing that it corresponds to EGFRvIII. Tyrosine phosphorylation of EGFRvIII was inhibited by concentrations of AG1478 of 1 μM or higher. This correlates well with the concentration of AG1478 required to inhibit ERK phosphorylation in the same samples. This, together with data described in the preceding paragraphs, demonstrates clearly that EGFRvIII activates ERKs in U87MGΔEGFR cells.

EGFRvIII has previously been shown to constitutively activate PI 3-kinase in 3T3 cells [21]. We also assessed the activation of PI 3-kinase by EGFRvIII in U87MGΔEGFR cells, a more clinically-relevant context. We used phosphorylation of PKB/akt at serine 473 as a marker of PI 3-kinase activity, as extensive research has shown that phosphorylation at this site is dependent on PI 3-kinase activity (reviewed in [22]). Fig. 1 shows the serine-473 phosphorylation status of PKB/akt in U87MGΔEGFR cells and U87MG cells. Phosphorylated PKB/akt was readily detected in U87MGΔEGFR cells. PKB/akt phosphorylation was blocked by treatment with AG1478, and again the concentration of AG1478 that inhibited PKB/akt phosphorylation corresponded well with the concentration that inhibited EGFRvIII tyrosine phosphorylation (Fig. 1B). PKB/akt phosphorylation was much lower or undetectable in U87MG cells. Levels of total PKB protein were the same in U87MGΔEGFR cells and U87MG cells, and were unaffected by AG1478 treatment. These results show that PI 3-kinase is constitutively activated by EGFRvIII in glioblastoma cells.

3.2. *EGFRvIII-induced ERK phosphorylation is blocked by PI 3-kinase inhibitors*

There have been reports that EGF receptor-mediated activation of ERKs is partially blocked by PI 3-kinase inhibitors in some cell types, but not in others. We asked whether EGFRvIII-induced phosphorylation of ERKS was blocked by PI 3-kinase inhibitors. U87MGΔEGFR and U87MG cells were treated for 20 min with the PI 3-kinase inhibitor wortmannin at a concentration of 100 nM, or with a second PI 3-kinase inhibitor, LY294002, used at a concentration of 50 μM. Analysis by Western blotting (Fig. 2) showed that these treatments completely blocked PKB phosphorylation without affecting levels of total PKB protein, indicating that PI 3-kinase was effectively inhibited. The treatments also did not significantly affect ERK protein levels (Fig. 2). However, the amount of phosphorylated ERKs was reduced to almost undetectable levels. In U87MG cells, EGFRvIII-independent ERK phosphorylation was also reduced by both PI 3-kinase inhibitors. It is likely that the effects of wortmannin and LY294002 seen in these experiments are due to direct inhibition

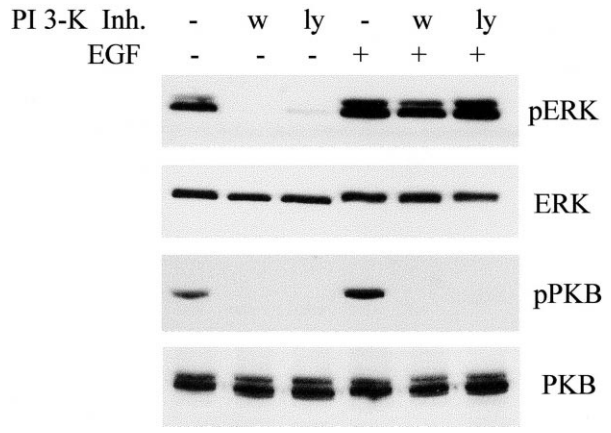

Fig. 2. PI 3-kinase inhibitor treatment in absence or presence of EGF. U87MGΔEGFR cells were treated with DMSO, 100 nM wortmannin (w) or 50 μM LY294002 (ly) for 20 min. Cells in indicated lanes were also treated for 5 min with 100 ng/ml EGF. (This was added for the last 5 min of the PI 3-kinase inhibitor treatment.) Total cell extracts were harvested and analyzed as in Fig. 1.

of PI 3-kinase. Wortmannin has been shown to inhibit other kinases [23]. However, the IC_{50} values for wortmannin inhibition of these enzymes are much higher than the concentration of wortmannin used here. Longer treatment (≥ 2 h) of U87MG and U87MGΔEGFR cells with wortmannin caused the cells to round up and detach from the plate. This was accompanied by a reactivation of ERKs, presumably secondary to these changes. For this reason, we did not feel that inhibition of PI 3-kinase by transfection of dominant negative versions of PI 3-kinase would provide useful information, as this approach involves longer term (i.e., 1–2 days) exposure of cells to PI 3-kinase inhibition.

3.3. ERK activation by normal EGFR in glioblastoma cells

We next compared the activation of ERKs by EGFRvIII in glioblastoma cells with ERK activation by normal EGF receptor. U87MGΔEGFR cells were treated with PI 3-kinase inhibitors or DMSO control as above, and then treated with 100 ng/ml EGF for 5 min to activate the normal EGF receptors present in these cells. As shown in Fig. 2, none of these treatments affected overall levels of ERKs. EGF treatment of U87MGΔEGFR cells did induce an increase in ERK phosphorylation above the levels seen in untreated cells. However, preexposure of cells to either wortmannin or LY294002 had little or no effect on the level of ERK phosphorylation seen in the presence of 100 ng/ml EGF, although PKB phosphorylation was completely blocked with these treatments. This is in marked contrast to the almost complete inhibition of the ERK phosphorylation induced by EGFRvIII. Similar results were obtained when lower levels of EGF (10 or 1 ng/ml) were used (data not shown). These data suggest that EGFRvIII uses a signaling pathway for ERK activation that is different from the pathway used by normal EGF receptor activated by EGF.

3.4. Levels of activated EGFRvIII and normal EGFR in glioblastoma cells

One possible explanation for this result comes from previous observations that low concentrations of activated EGF receptors activate ERK by a wortmannin-sensitive pathway, whereas high levels of activated EGF receptor use a pathway that is predominantly wortmannin-insensitive [15]. Analysis by flow cytometry shows that U87MG cells express 2×10^5 normal EGF receptors per cell and that the U87MGΔEGFR cells express between 4×10^5 and 2×10^6 EGFRvIII per cell [7,24]. EGFRvIII is therefore present at higher levels than wild-type EGF receptor in U87MGΔEGFR cells. However, it has been reported that not all EGFRvIII is tyrosine-phosphorylated in these cells. To directly compare levels of activated receptors in U87MGΔEGFR cells, we analyzed Western blot samples for levels of EGF receptor total protein and phosphotyrosine levels. Analysis of samples with an antibody that recognizes both normal EGF receptor and EGFRvIII (raised against a sequence in the EGF receptor cytoplasmic domain) show that levels of normal EGF receptor (175 kDa) and EGFRvIII (two bands of approximately 150 and 140 kDa, as reported previously [7]) were the same in each sample and were unaffected by any of the treatments (Fig. 3). Analysis of phosphotyrosine levels in EGFRvIII and normal EGF receptor in U87MGΔEGFR cells treated with PI 3-kinase inhibitors showed that PI 3-kinase inhibitors had no effect on the levels of phosphotyrosine in EGFRvIII. Thus the blockade of EGFRvIII-induced ERK phosphorylation by PI 3-kinase inhibitors is not due interference with receptor tyrosine phosphorylation. Treatment with EGF resulted in the appearance of a higher molecular mass phospho-

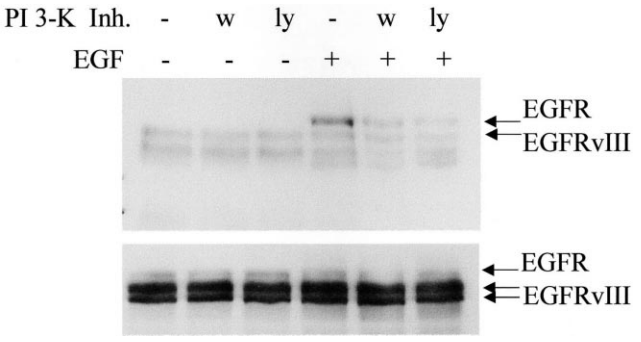

Fig. 3. (A) Tyrosine phosphorylation of normal and mutant EGF receptors. U87MGΔEGFR cells were treated with DMSO, wortmannin (w) or LY294002 (ly) as in Fig. 3. Cells in indicated lanes were treated with EGF, also as in Fig. 3. Total cell extracts were analyzed for phosphotyrosine, normal EGF receptor and EGFRvIII by Western blotting.

tyrosine-labeled band corresponding to normal EGF receptor. In cells treated with EGF, we reproducibly saw a small decrease in EGF receptor tyrosine phosphorylation when cells were preincubated with PI 3-kinase inhibitors. Although the mechanism for this is unknown, it is possible that PI 3-kinase directly or indirectly regulates the activity of a phosphotyrosine phosphatase that can use normal EGF receptor as a substrate. The levels of phosphotyrosine in EGFRvIII and normal EGF receptor in cells treated with PI 3-kinase inhibitors were very similar under these experimental conditions. This shows that the preferential use of a PI 3-kinase-sensitive pathway by EGFRvIII is not due to a lower level of activated EGFRvIII compared to activated normal EGF receptor.

3.5. Role of MEK in ERK activation by EGFRvIII

Most studies have shown that ERK phosphorylation is directly mediated by the protein kinase MEK. However, MEK-independent activation of ERK has been described [25]. We tested whether ERK phosphorylation was inhibited by the specific MEK inhibitor PD98059. We found that ERK phosphorylation induced both by EGFRvIII and normal EGF receptor was completely blocked by this inhibitor (Fig. 4A). From this it can be concluded that the different ERK activation pathways used by normal and mutant EGF receptors converge at or above the level of MEK.

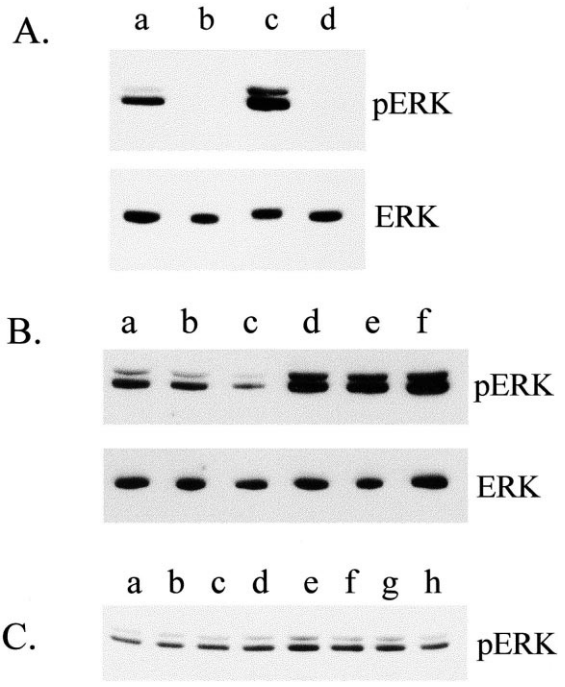

Fig. 4. Effects of MEK, phospholipase C and PKC inhibitors on ERK phosphorylation. (A) U87MGΔEGFR cells were treated for 90 min with DMSO (lanes a,c) or the MEK inhibitor PD98059 (at a final concentration of 50 μM). Cells in lanes c and d were treated for 5 min with EGF. (B) U87MGΔEGFR cells were treated with DMSO (lanes a,d), U73433 (lanes b,e) or U73122 for 15 min. Cells in lanes d–f were treated for 5 min with EGF. (This was added for the last 5 min of the PLC inhibitor treatment.) (C) U87MGΔEGFR cells were treated for 1 h with 0, 10, 100 or 500 nM of GÖ6976 (lanes a–d) or 0, 10, 100 or 500 nM of calphostin C (lanes e–h). Total cell extracts were harvested and analyzed as in Fig. 1.

3.6. Role of phospholipase C in ERK activation by EGFRvIII

We next assessed a possible role for phospholipase C (PLC) in ERK activation by EGFRvIII. U87MGΔEGFR cells were treated for 15 min with the aminosteroid phospholipase C inhibitor U73122 [26]. For controls, cells were treated with DMSO alone, or with U73433, an inactive analogue of U73122. This treatment has been shown to inhibit 80–90% of PLC-γl activity in CHO cells [27]. None of the treatments affected levels of total ERK protein. However, U73122 treatment did cause a marked inhibition of ERK phosphorylation (Fig. 4B). This inhibition was not seen with U73343, or with DMSO alone. When normal EGF receptors in U87MGΔEGFR cells were activated with EGF either in the absence of presence of U73122, no differences in the level of ERK phosphorylation were observed.

3.7. Effects of conventional and novel protein kinase C inhibitors on ERK activation by EGFRvIII

The results with the U73122 inhibitor suggested a role for phospholipase C in activation of ERKs by EGFRvIII. One way that phospholipase C could activate ERKs would be by generation of the second messenger diacylglycerol, which in turn could activate the conventional or novel PKC family members [28]. There is extensive evidence that these PKCs can activate ERKs, although the exact mechanism by which this occurs is still not known. To determine

if the conventional PKCs played a role in ERK activation by EGFRvIII, we treated cells with GÖ6976, a selective inhibitor of PKCα and PKCβI [29]. We saw no inhibition of ERK phosphorylation over a range of inhibitor concentrations from 10 to 500 nM (Fig. 4C, lanes a–d). We also tested the effect of calphostin C, an inhibitor of both conventional and novel PKCs that acts by inhibiting diacylglycerol binding to these enzymes [30]. Again we saw no effect of this inhibitor over a range of concentrations from 0 to 500 nM (Fig. 4C, lanes e–h). These results suggest that the neither the conventional nor novel PKC enzymes have an essential role in ERK activation induced by EGFRvIII.

4. Discussion

We have shown that both EGFRvIII and normal EGF receptor induce the phosphorylation of ERKs in glioblastoma cells. However, they appear to do so by very different mechanisms. Thus EGFRvIII-induced ERK phosphorylation was sensitive to two different PI 3-kinase inhibitors, while ERK phosphorylation induced by EGF-activated normal EGF receptor was insensitive to these inhibitors. This difference was seen under conditions where amounts of activated normal and mutant EGF receptors were at a similar high level. Previous work by others has shown that normal EGFR is able to activate ERKs by a wortmannin-sensitive pathway, but only at very low levels of activated receptor [15]. There is considerable evidence that different kinetics of ERK activation can result in completely different cellular responses [13] and that different signaling pathways are used to elicit these different kinetics of activation [25]. Thus an understanding of the signaling pathway used by EGFRvIII to activate ERKs may help explain its potent ability to enhance tumor growth in nude mice.

We also found that EGFRvIII activation of ERKs was blocked by an inhibitor of PLC. Again this was not seen with ERK activation by normal EGFR. Although the inhibitor used here has not been demonstrated to be specific for PLC-γ1, this PLC would seem likely to be the relevant target, given its well known role in receptor tyrosine kinase signaling [31]. It has been shown that activation of PLC-γ1 involves both the interaction of its src homology 2 domains with receptor phosphotyrosine and also the interaction of its pleckstrin homology domains with phosphatidylinositol 3,4,5-trisphosphate, the product of PI 3-kinase. Inhibition of PI 3-kinase by wortmannin blocks PLC-γ translocation to the plasma membrane and partially blocks the production of inositol 1,4,5-trisphosphate by this enzyme in cells treated with PDGF [32]. Thus the inhibition of ERK phosphorylation by wortmannin in U87MGΔEGFR cells might be due, in part or in whole, to its inhibitory effect on PLC-γ activation.

PLC-γ catalyzes the production of the second messengers diacyglycerol and inositol 1,4,5-trisphosphate, with the latter inducing the release of calcium from intracellular stores. This can in turn lead to activation of either the conventional PKCs (activated by Ca^{2+} and diacyglycerol) or novel PKCs (activated by diacylglycerol alone). Members of both these PKC families are able to activate ERKs [33], although the mechanism by which they do this is not clear [34]. However, ERK activation induced by EGFRvIII was not blocked by several different PKC inhibitors that we tested, suggesting that these enzymes do not mediate ERK activation by EGFRvIII. One possible explanation is that increases in cytosolic Ca^{2+} could activate ERKs via a pathway other than the PKC pathway; as one possibility, the Ca^{2+}/calmodulin-dependent protein kinases have also been shown to mediate activation of ERKs in some cell types [35]. However, we also have not observed any inhibition of ERK phosphorylation when U87MGΔEGFR cells were treated with the Ca^{2+}/calmodulin dependent protein kinase inhibitor KN-62[1]. Alternatively, the effects of U73122 could be due to inhibition of some enzyme other than another PLC-γ. ERK activation induced by engagement of integrin/fibronectin receptors has also been shown to require PI 3-kinase: this has been shown to be due to PI 3-kinase-mediated activation of raf1 via p21-activated protein kinase [36]. We are currently investigating whether EGFRvIII also uses a similar pathway to activate ERKs.

There are several possible reasons why EGFRvIII might use a different pathway for ERK activation.

[1] Lorimer and Lavictoire, unpublished observations.

One possibility is that EGFRvIII preferentially heterodimerizes with another member of the erbB receptor family in these cells. We feel this is unlikely, as it has been reported that U87MG cells contain undetectable levels of other erbB family members [37]. A second possibility is that EGFRvIII adopts a different conformation in its cytoplasmic domain that directly alters its affinity for specific adaptor molecules. Several lines of evidence suggest that EGFRvIII might adopt a different conformation of its cytoplasmic domain compared to normal EGFR: these include the fact that EGFRvIII is downregulated much less rapidly than normal EGFR [9], and the reported differential sensitivity of EGFRvIII to tyrosine kinase inhibitors compared to normal EGFR [19]. A third possibility is that the slow internalization of EGFRvIII [9] allows it to engage this pathway, while normal EGFR is unable to engage this pathway efficiently because of its rapid downregulation by internalization. This model implies that there are differences in the association and dissociation kinetics of different adaptor proteins. Further studies comparing the interaction of normal and mutant EGFR with adaptor proteins should help to determine which of these mechanisms is correct.

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